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Hormonal activation of *let-7-C* microRNAs via EcR is required for adult *Drosophila melanogaster* morphology and function

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SUMMARY

Steroid hormones and their nuclear receptors drive developmental transitions in diverse organisms, including mammals. In this study, we show that the *Drosophila* steroid hormone 20-hydroxyecdysone (20E) and its nuclear receptor directly activate transcription of the evolutionarily conserved *let-7-complex* (*let-7-C*) locus, which encodes the co-transcribed microRNAs miR-100, let-7 and miR-125. These small RNAs post-transcriptionally regulate the expression of target genes, and are required for the remodeling of the *Drosophila* neuromusculature during the larval-to-adult transition. Deletion of three 20E responsive elements located in the *let-7-C* locus results in reduced levels of *let-7-C* microRNAs, leading to neuromuscular and behavioral defects in adults. Given the evolutionary conservation of *let-7-C* microRNA sequences and temporal expression profiles, these findings indicate that steroid hormone-coupled control of *let-7-C* microRNAs is part of an ancestral pathway controlling the transition from larval-to-reproductive animal forms.

KEY WORDS: *let-7-complex*, *let-7-C*, *let-7*, MicroRNA, Ecdysone receptor, EcR

INTRODUCTION

Temporal cell fate transitions during *Caenorhabditis elegans* development are controlled by the heterochronic pathway, which includes members of the lin-4 and let-7 family of short noncoding microRNAs (miRNAs) [for a recent review of the heterochronic pathway, see Ambros (Ambros, 2011)]. These miRNAs are expressed during discrete stages of worm development, and their insect and vertebrate orthologs display analogous temporal patterns of expression (Pasquinelli et al., 2000; Sempere et al., 2003). The *Drosophila melanogaster* let-7 and lin-4 orthologs, for example, are undetectable during embryonic and larval development, but are highly expressed during pupal development (Sempere et al., 2002; Bashirullah et al., 2003; Sempere et al., 2003), ensuring the appropriate differentiation of adult tissues (Caygill and Johnston, 2008; Sokol et al., 2008). Although these temporal expression profiles indicate the conserved function of heterochronic miRNAs in diverse animals, it has been unclear whether they are the result of analogous regulatory mechanisms.

Activation of *C. elegans* heterochronic miRNAs is influenced by environmental conditions, including food availability and population density, and is controlled in part by steroidal dafachronic acids (DAs). These steroid hormones are produced in favorable environmental conditions and in response to the transforming growth factor β (TGFβ) and insulin signaling (IGF) pathways (reviewed by Fielenbach and Antebi, 2008). In the presence of DAs, the Abnormal Dauer Formation nuclear hormone receptor DAF-12 activates the promoters of two of the four members of the let-7 family, *miR-84* and *miR-241*, synchronizing developmental progression with environmental conditions (Bethke et al., 2009; Hammell et al., 2009). The postembryonic activation of *lin-4* in response to feeding similarly links the heterochronic pathway to environmental conditions (Lee et al., 1993; Feinbaum and Ambros, 1999), although the transcriptional mechanism controlling this nutrient-dependent expression is unknown.

Steroid hormones may also directly control the expression of *Drosophila* let-7 and miR-125, the respective *Drosophila* orthologs of let-7 and lin-4. These two miRNAs are co-transcribed along with a third miRNA, miR-100, from a single locus called the *let-7-complex* (*let-7-C*) (Fig. 1A), and all three processed miRNAs are initially detected at the beginning of the larval-to-pupal transition (Sempere et al., 2002; Bashirullah et al., 2003; Sempere et al., 2003; Sokol et al., 2008). Many genes are regulated at this time by pulses of the 20-hydroxyecdysone (20E) steroid hormone (Andres and Thummel, 1994), the production of which is controlled by both the TGFβ and IGF pathways in a fashion similar to DAs (reviewed by Tennessen and Thummel, 2011). 20E activates the transcription of target genes by binding to a heterodimeric protein receptor composed of two members of the nuclear receptor family, Ecdysone Receptor (EcR) and the RXR ortholog Ultraspiracle (Usp) (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993; Riddiford et al., 2000; King-Jones and Thummel, 2005). The developmental expression profile of miR-100, let-7 and miR-125 therefore suggests that the *let-7-C* locus may be a direct target of the 20E/EcR signaling pathway.

Previous reports, however, have provided ambiguous results regarding the control of *let-7-C* miRNAs by 20E and EcR. Although 20E induces the expression of all three miRNAs in *Drosophila* tissue culture via EcR, this activation may not be direct as the appearance of *let-7-C* miRNAs occurs well after that of other known direct targets of 20E/EcR (Sempere et al., 2002; Sempere et al., 2003; Garbuzov and Tatar, 2010). Furthermore *let-7-C* miRNAs are still activated during larval development, even after EcR expression is knocked down by RNA interference (Bashirullah et al., 2003), implicating a role for other steroid hormone receptors in vivo. The mechanisms controlling *let-7-C* expression therefore remain unclear. We re-address this topic here, focusing on the expression of the previously identified ~2.5 kb primary *let-7-C* transcript (pri-let-

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7-C) (Sokol et al., 2008). This primary transcript is first processed into three ~70 nucleotide hairpins (pre-miR-100, pre-let-7, pre-miR-125) and these pre-miRNAs subsequently release the mature ~21 nucleotide miRNAs. Our analysis of pri-let-7-C as a reporter of the transcriptional activity of the *let-7-C* locus indicates that *let-7-C* miRNAs are the direct target of 20E/EcR.

MATERIALS AND METHODS

Fly strains and techniques

UAS-EcR-RNAi, *UAS-EcRA-W650A*, *UAS-EcRA*, *UAS-EcRB1* and *UAS-EcRB2* transgenic fly strains were obtained from the Bloomington Stock Center. *sg-Gal4; Sgs3-GFP* was a gift from Andrew J. Andres (Costantino et al., 2008). The let-7-$Cp^{12.5kb}$::LacZ, let-7-$Cp^{12.5kb\Delta EcRE1\text{-}3}$::LacZ, let-7-$Cp^{3.3kb}$::LacZ, let-7-$Cp^{3.3kb\Delta EcRE1\text{-}3}$::LacZ, let-7-$Cp^{3.3kb}$::cDNA, and let-7-$Cp^{3.3kb\Delta EcRE1\text{-}3}$:: cDNA transgenes were inserted into the PBac{y[+]-attP-3B}VK00033 landing site by Rainbow Transgenic Services (CA, USA). Standard genetic crosses were used to obtain flies of indicated genotypes. Behavioral analyses were performed as previously described (Sokol et al., 2008). Experiments involving staged animals included animals of the following ages: embryonic (E) stage, 12-16 hours after egg laying (AEL); L1 stage, 24-27 hours AEL; L2 stage, 48-51 hours AEL; eL3, 72-75 hours AEL. Wandering larvae were staged according to the food remaining in their gut (Maroni and Stamey, 1983; Andres and Thummel, 1994). Pre-pupae and pupae were staged in hours after pupariation (APF). Staging relative to the second-to-third ecdysis was performed using a protocol adapted from Warren et al. (Warren et al., 2006).

To generate flp-out clones, *yw hsFLP*^{122}; *let-7-*$Cp^{3.3kb}$*::LacZ* virgins were crossed to males bearing Act5c>CD2>Gal4, UAS-RFP on the third chromosome and either UAS-EcR-RNAi or UAS-B1-DeltaC_{655}.W650A on the second chromosome. After 2 days of egg laying, adults were removed and the progeny were aged for 1 more day, heat shocked (38°C for 5-10 minutes), and further maintained at 25°C for 3-5 days prior to dissection. Imaginal discs and CNS were collected from animals of the indicated stages and processed for immunostaining.

RNA isolation, northern blot analysis and semi quantitative RT-PCR

Total RNA was extracted from animals or cell lines using Tri Reagent (Molecular Research Center). Northern blots were conducted as described by Sempere et al. (Sempere et al., 2003). Radioactive labeled StarFire (Integrated DNA Technologies) oligonucleotide probes used for miRNA detection are listed in supplementary material Table S1. Radioactive signals were quantified with ImageQuant software (Molecular Dynamics). The relative levels of miRNA transcripts were represented as the ratio of the miRNA and U6 snRNA signals. For RT-PCR, 1.5 μg RNA was used to generate cDNA using Superscript III reverse transcriptase (Invitrogen). Random hexamers were used for cDNA synthesis. Diluted α-^{32}P-dATP was included in the PCR cocktail to allow relative quantitation of transcripts. All PCR products were normalized to Actin 5c. Thirty-two cycles of PCR amplification were used to amplify endogenous and reporter *let-7-C* constructs, and 22-25 cycles were used for amplifying Actin 5c, EcR, E75, Sgs3, IMP E1, Lsp1α and Lsp 1β. Primers used for PCR are listed in supplementary material Table S1.

Cell culture, transient tranfections and luciferase assays

Drosophila Kc-167 and L57-3-11 cells were cultured in CCM3 at 23°C. 20E (Sigma) was added to a final concentration of 1×10^{-6} M for reporter gene assays. For analysis of endogenous transcripts, 5×10^{-6} M 20E was used. Control cells were treated with an equal volume of solvent (ethanol). Transfections of Kc-167 cells with the luciferase reporter plasmids were performed using Effectene (Qiagen). For 1.5×10^{6} cells, 125 ng of S-188-cc-Rluc and 250 ng of the enhancer-driven S-188-cc-Luc was used. A 1:5 ratio of the reporters to the EcR-DN or EcR expression plasmids was maintained wherever needed [pCMA EcR B1 W650A, pCMA-EcR A, pCMA EcR-B1 and pCMAEcR-B2 (Hu et al., 2003)]. 20E (10^{-5}M) in ethanol carrier was added 3 hours after the transfection (Hu et al., 2003) and cells were harvested for reporter assay 45-49 hours after the transfection. Luciferase plasmids were analyzed using the Dual-Luciferase reporter system (Promega), with firefly luciferase as the primary reporter and Renilla luciferase as an internal transfection control (Hu et al., 2003). Data represent the mean of at least three independent experiments unless otherwise stated, with error bars indicating s.e.m.

Electrophoretic mobility shift assays (EMSAs)

Proteins for all EMSAs were generated by in vitro transcription and translation reactions using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Proteins were incubated with binding buffer [90 mM KCl, 10% glycerol, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*9-2-ethanesulfonic acid; pH 7.5), 0.9 mM dithiothreitol, 1 mM EDTA and 1 mM Tris (pH 7.5)] on ice for 10 minutes with 1 μg of non-specific competitor poly(dI-dC)-poly(dI-dC). Approximately 1 ng of [^{32}P] dATP probe was added to the reaction and incubated at room temperature for 25 minutes. Probes were labeled by a fill-in reaction using the Klenow fragment. The complexes formed were loaded on a pre-run native polyacrylamide gel [6% (wt/vol) 29:1 acrylamide-bis-acrylamide, 0.5×Tris-borate-EDTA]. Gels were resolved and analyzed on a Phosphorimager.

The Kc-167 nuclear extracts were prepared from cells treated with 20E for 6 hours using a previously described protocol (Cakouros et al., 2004). For supershift assays, 1 μl of monoclonal EcR-B1 (AD4.4), EcR common(AG10.2) or EcR-A(15G1A) antibody was added to the nuclear extracts or in vitro translated proteins, and incubated on ice before the addition of labeled probe.

Plasmid construction

The pP{W8, *let-7-C*} encoding the *let-7-C* locus (Sokol et al., 2008) was used as the backbone for making luciferase reporter constructs, *lacZ* transgenes and rescue transgenes. All enhancer luciferase constructs were generated by PCR cloning into the S-188-cc-Luc vector (Hu et al., 2003). PCR products were verified by sequencing. Reporters containing minimal EcRE sequences were generated by cloning annealed oligonucleotides. Detailed description of *lacZ* and rescue transgene construction is available upon request. The primers used for these clonings are listed in supplementary material Table S1.

Immunostaining and microscopy

Tissues were fixed for 30-45 minutes in 4% buffered formaldehyde, washed in phosphate-buffered saline with 0.3% Triton X-100 (PBT), blocked in 5% donkey serum in PBT (SPBT) and incubated in primary antibody overnight at 4°C. Indicated dilutions of the following primary antibodies were made in SPBT: rabbit anti-dsRed (Clontech Laboratories; 1:60,000), mouse anti-EcR-A (15G1A ascites; 1:1000), mouse anti-EcR-B1 (AD4.4; 1:1000), mouse anti-EcR-common (AG10.2; 1:1000) and mouse anti-β-gal (Promega; 1:1000). After rinsing, tissues were blocked again and then incubated for 2 hours in secondary antibodies made in SPBT. Secondary antibodies included goat anti-rabbit Alexa 568 (1:5000) (Molecular Probes), goat anti-chicken Alexa-488 (Molecular Probes), goat anti-mouse Alexa-488 (1:500) (Molecular Probes) and rhodamine phalloidin (1:2000, Sigma). Tissues were mounted in 90% glycerol-DABCO. All experiments were imaged on a Leica SP5 confocal microscope (Light Microscopy Imaging Center, Indiana University, USA).

RESULTS

pri-let-7-C is first detected in mid-third instar larvae

To characterize the *let-7-C* transcriptional profile during *Drosophila* development, we used semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) to detect the pri-let-7-C transcript in total RNA extracted from staged animals (Fig. 1B). The primary transcript was first detected in third instar larvae (L3) that were collected ~18 hours prior to puparium formation (–18 hours APF), and was detected at approximately constant levels throughout the remainder of development. To correlate the expression of pri-let-7-C with processed *let-7-C*

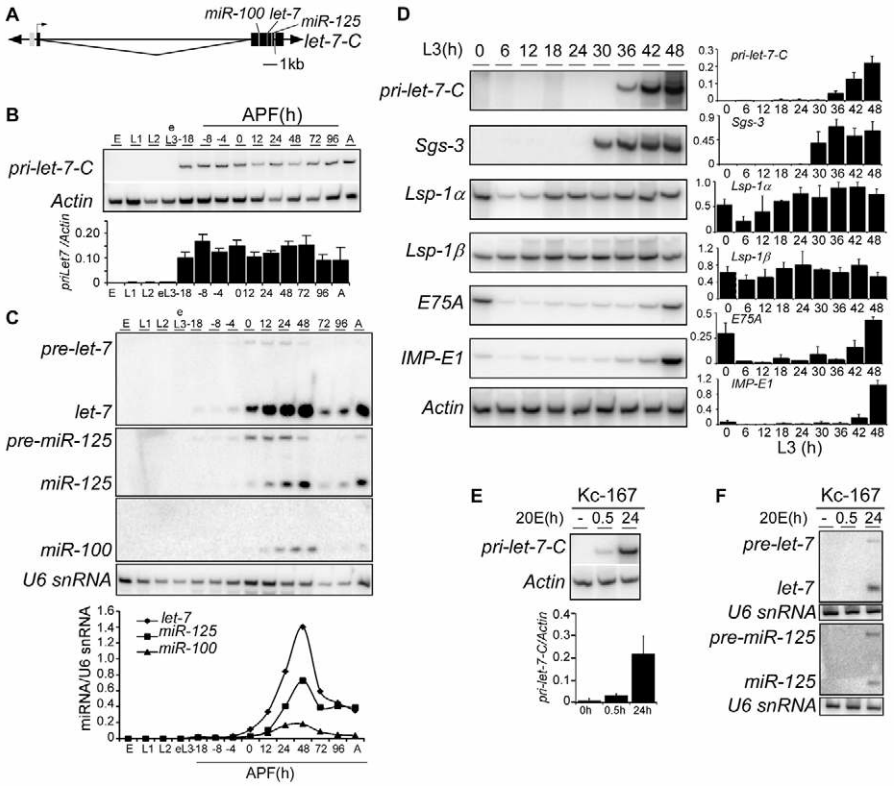

Fig. 1. Developmental profile and 20E induction of pri-let-7-C transcript. (**A**) Schematic representation of the *let-7-C* locus that spans 17,400 bp of genomic DNA and encodes three evolutionary conserved miRNAs, miR-100, let-7 and miR-125 (shown as vertical gray lines). (**B**) Developmental profile of pri-let-7-C transcript, as detected by semi-quantitative RT-PCR of total RNA extracted from staged embryos (E), first (L1), second (L2), third instar larvae [early L3 (eL3), –18 hours APF, –8 hours APF, –4 hours APF], prepupae, pupae and adults using oligos that spanned the first intron of *let-7-C*. Actin 5c transcript was analyzed as a control for loading. Histograms depicting the normalized levels of the RT-PCR products from three independent experiments are represented below the gel images. (**C**) Developmental profile of mature let-7, miR-125 and miR-100 miRNAs, as detected by northern blot analysis of total RNA extracted from same developmental stages as B using antisense let-7, miR-125 and miR-100 probes. U6 snRNA was analyzed as a control for loading. The graph below the gel image depicts the levels of processed let-7-C miRNAs relative to U6 snRNA. (**D**) Developmental profile of pri-let-7-C transcript, as detected by semi-quantitative RT-PCR of total RNA extracted from staged third instar larvae. E75A, ImpE1, Sgs3, Lsp 1α and Lsp1β transcripts were also analyzed, and Actin 5c was used as a loading control. Histogram plots representing the levels of the different transcripts relative to Actin 5c from three independent experiments are represented on the right. (**E**) 20E induction of pri-let-7-C transcript, as detected by semi-quantitative RT-PCR of total RNA extracted from Kc-167 cells treated with 5×10^{-6} M 20E for 0.5 and 24 hours. Untreated cells are shown in the left-hand lane. Histogram plot depicting pri-let-C levels relative to Actin 5c (n=3) is shown below the gel. (**F**) Northern blot detection of mature let-7 and miR-125 miRNAs in the same samples as E. U6 snRNA was used as a loading control for northern blot analysis.

miRNAs, northern blots of the same developmentally staged RNA samples were probed for miR-100, let-7 and miR-125 (Fig. 1C). let-7 and miR-125 were first detected at –18 hours APF, while miR-100 was detected at –4 hours APF (Fig. 1C). The mature *let-7-C* miRNAs displayed a more dynamic expression profile than pri-let-7-C: they rose during the first half of metamorphosis, peaked at 48 hours APF and then declined precipitously (compare Fig. 1B with 1C). The delayed onset and reduced expression of miR-100 relative to let-7 and miR-125, as detected by our northern blots are consistent with the reduced relative expression of miR-100 detected by deep sequencing analyses (Berezikov et al., 2011). These observations indicate that the three *let-7-C* miRNAs are differentially processed, and is consistent with data that miR-100 is edited by the *Drosophila* Adenosine Deaminase, whereas let-7 and miR-125 are not (Berezikov et al., 2011). Taken together, these data indicated that the *let-7-C* locus is activated transcriptionally in mid-third instar larvae, and that the dynamic expression of mature *let-7-C* miRNAs reflects post-transcriptional processing of pri-let-7-C.

pri-let-7-C is induced by Ecdysone

To determine precisely the timing of *let-7-C* transcriptional activation during the L3 stage, RNA was extracted from third instar larvae staged relative to the second-to-third larval ecdysis and analyzed by RT-PCR (Fig. 1D). Transcripts previously shown to be expressed at different time points in the third instar were used as controls, and included Sgs3, Lsp1α, Lsp1β, E75A and IMP E1 (Andres et al., 1993). Pri-let-7-C transcript was first detected at 36 hours after larval ecdysis (Fig. 1D), at approximately the time when larvae stop feeding and prepare to pupariate. Pri-let-7-C was detected shortly after Sgs3, a glue gene transcript known to be induced by the mid-third larval instar 20E peak (Guay and Guild, 1991; Andres et al., 1993). To test whether *let-7-C* could also be induced by 20E, pri-let-7-C expressoin was examined in the 20E responsive embryonic Kc-167 cell line (Echalier and Ohanessian, 1969; Savakis et al., 1980). Although undetectable in untreated Kc-167 cells, moderate levels of the primary transcript were detected 30 minutes after exposure to 20E and increased sharply by 24 hours after hormone treatment (Fig. 1E). The accumulation of

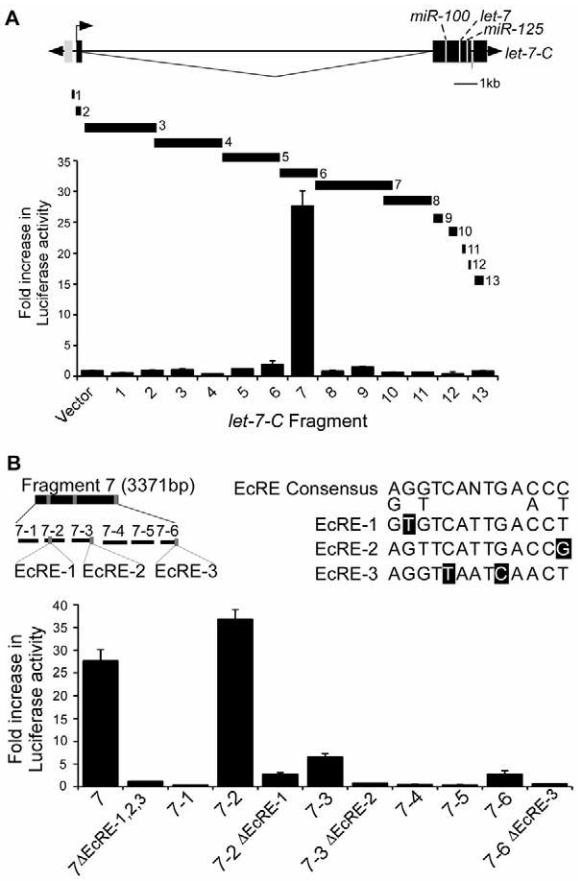

Fig. 2. *let-7-C* intron 1 encodes three Ecdysone response elements (EcREs). (**A**) Fold increase in luciferase activity of 20E-treated Kc-167 cells transfected with a series of luciferase reporter constructs containing fragments (1-13) spanning the *let-7-C* locus. Location of each fragment is shown relative to *let-7-C* locus schematic (top panel). Fold induction of each construct (vertical axis) measured as luciferase activity of 20E-treated versus untreated samples. (**B**) Fold increase in luciferase activity of 20E-treated Kc-167 cells transfected with a series of six firefly luciferase reporter constructs containing fragments (7-1 to 7-6) spanning 20E inducible fragment 7 identified in A. 20E-inducible fragments 7-2, 7-3 and 7-6 contain EcRE-1, EcRE-2 and EcRE-3 sequences, respectively (shown on the right relative to EcRE consensus sequence). Reporter assays were also performed on fragments deleted of the EcREs. The histogram plot representing fold increase in luciferase activity±s.d. (n=3) is shown.

processed let-7 and miR-125 RNAs was slower, as they were only detected in the 24 hour time-point (Fig. 1F), as has been shown previously (Bashirullah, 2003). This delay probably reflected the post-transcriptional processing of *let-7-C* miRNAs. The rapid accumulation of pri-let-7-C indicated that the *let-7-C* locus is a direct transcriptional target of 20E.

The *let-7-C* locus contains three Ecdysone response elements (EcREs)

To identify cis-regulatory elements controlling pri-let-7-C levels, fragments of genomic DNA spanning the ~18 kb *let-7-C* locus were tested for their ability to confer 20E responsiveness to a luciferase reporter gene (Fig. 2A). Constructs were transiently transfected into Kc-167 cells, and luciferase activity was measured 48 hours after 20E treatment. Of the 13 fragments tested, a single 3371 bp fragment (Fragment 7, Fig. 2A) from intron 1 conferred 20E responsiveness. This fragment was subdivided into six ~500 bp fragments (Fragments 7-1 to 7-6, Fig. 2B), which were subjected to the same analysis. Only fragments 7-2, 7-3 and 7-6 conferred a 20E response, and they mediated 37-, 6.5- and 2.7-fold increases in luciferase activity upon 20E treatment, respectively (Fig. 2B). This analysis indicated that the *let-7-C* locus contains at least three EcREs that are clustered within 3.3 kb of one another.

To map these cis-regulatory sequences in more detail, we bioinformatically searched fragments 7-2, 7-3 and 7-6 for the EcRE consensus sequence motif (Cherbas et al., 1991). This 13 bp regulatory element is bound by a EcR/Usp heterodimeric complex, which then activates transcription of target genes when liganded to 20E (Koelle et al., 1991; Yao et al., 1992; Thomas et al., 1993; Yao et al., 1993). Fragments 7-2, 7-3 and 7-6 each contained a sequence that matched at least 11 bp of the consensus EcRE (Fig. 2B), and that was conserved across 12 *Drosophila* species (supplementary material Fig. S1). To determine whether these putative EcREs were responsible for the 20E-responsiveness of fragments 7-2, 7-3 and 7-6, mutated versions of each fragment that lacked the putative EcRE were tested in the luciferase assay. Each mutated fragment failed to confer 20E induction of luciferase activity (Fig. 2B). These data indicated that the three sequences we identified bioinformatically functioned as bona fide EcREs, and were named EcRE-1 to EcRE-3, respectively. Furthermore, deletion of all three EcREs from the 3.3 kb fragment 7 completely abolished its 20E responsiveness (Fig. 2B), and indicated that the 20E responsiveness of the *let-7-C* locus in Kc-167 cells is mediated by these EcREs.

To further characterize EcRE-1 to EcRE-3, we tested whether each sequence was alone sufficient to confer 20E-inducible transcription. Reporters containing wild-type versions of each EcRE were prepared and tested for 20E-dependent luciferase activity (Fig. 3A). All three EcREs conferred 20E induction of luciferase activity, and EcRE-2, EcRE-1 and EcRE-3 mediated a 15-, 12- and 8-fold upregulation, respectively (Fig. 3B, black bars). Mutations in one half-site of the palindromic EcRE sequence abolished luciferase activation in response to 20E treatment (Fig. 3B, gray bars). These data demonstrated that three EcRE motifs located within a ~3.3 kb fragment of the first intron of the *let-7-C* locus are necessary and sufficient for 20E-induced transcription in cell culture, and suggested that EcR is a direct transcriptional activator of pri-let-7-C.

EcRE-1 to EcRE-3 are required for proper spatiotemporal *let-7-C* locus expression

To characterize the role of the *let-7-C* EcREs in vivo, we first tested whether EcRE-containing fragments could recapitulate the expression pattern of the endogenous *let-7-C* locus. We prepared *lacZ* transcriptional reporters that contained almost the entire first intron of *let-7-C* or just the 20E-responsive 3.3 kb Fragment 7 (Fig. 2A). The resulting *let-7-Cp*$^{12.5kb}$*::LacZ* and *let-7-Cp*$^{3.3kb}$*::LacZ* transgenes (Fig. 4A) were each inserted into identical genomic locations using the ϕC31 system (Bischof et al., 2007), which allowed direct comparison of their expression patterns. Consistent with the semi-quantitative RT-PCR analysis, the *let-7-Cp*$^{12.5kb}$*::LacZ* was first detected in the central nervous systems (CNS) of –18 hour third instar larvae and was subsequently detected in the imaginal discs of –8 hour third instar larvae (Fig. 4B; supplementary material Fig. S2). The spatial expression of *let-7-Cp*$^{12.5kb}$*::LacZ* resembled the previously reported expression of *let-7-C*^{GKI} in which Gal4 had been inserted in the *let-7-C* locus (Sokol et al., 2008), indicating that the

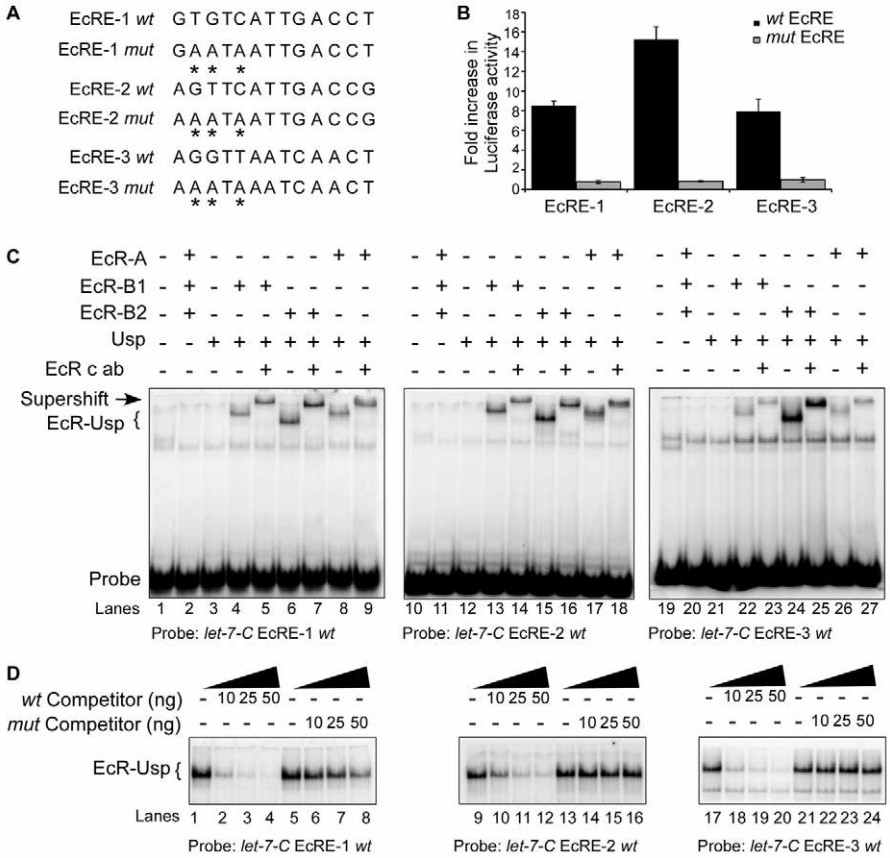

Fig. 3. *let-7-C* EcREs bind EcR-Usp heterodimer. (**A**) Wild-type and mutant *let-7-C* EcRE-1, EcRE-2 and EcRE-3 sequences. Asterisks indicate residues changed in mutant EcREs. (**B**) Fold increase in luciferase activity of 20E-treated Kc-167 cells transfected with luciferase reporters containing wild-type and mutant EcRE sequences shown in A. Fold increase was measured as luciferase activity of 20E-treated versus untreated samples. Histograms indicate fold increase of wild-type (black bars) and mutant (gray bars) EcRE-containing constructs from three independent experiments. (**C**) Electrophoretic mobility shift assay of *let-7-C* EcRE-1, EcRE-2 and EcRE-3 probes incubated with in vitro translated EcR isoforms (EcR-A, EcR-B1 and EcR-B2) and Usp proteins in the presence or absence of an anti-EcR-c antibody. Complexes were resolved on native polyacrylamide gels. EcR-Usp complexes and supershifted EcR-Usp complexes are indicated with a bracket and arrow, respectively. (**D**) In vitro translated EcR and Usp proteins were incubated with the wild-type EcRE probes for 30 minutes in the presence of increasing amounts of wild-type EcRE or mutant EcRE competitor oligonucleotides. Complexes were resolved on native polyacrylamide gels.

first *let-7-C* intron contains crucial regulatory elements. The *let-7-Cp$^{3.3kb}$::LacZ* reporter was detected in the CNS, ring gland and imaginal discs at approximately –18 hours APF followed by expression in salivary glands at puparium formation (Fig. 4B; supplementary material Fig. S3; data not shown). The 3.3 kb reporter displayed a much stronger staining pattern in third instar tissues when compared with the 12.5 kb intron reporter, suggesting that it lacked cis-acting repressor modules that were present in the 12.5 kb fragment. Together, these data demonstrated that the first intron of *let-7-C* contains regulatory elements that direct its correct temporal and spatial expression pattern.

To determine the role of the EcREs in driving these expression profiles, we generated two additional transgenes in which each of the 13 bp EcRE sequences were deleted (Fig. 4A). These *let-7-Cp$^{12.5kb\Delta EcRE1\text{-}3}$::LacZ* and *let-7-Cp$^{3.3kb\Delta EcRE1\text{-}3}$::LacZ* transgenes were inserted into identical genomic locations to the wild-type versions, allowing direct comparison. In contrast to the wild-type *lacZ* reporter, expression of the mutated *let-7-Cp$^{12.5kb\Delta EcRE1\text{-}3}$::LacZ* was not detected in the imaginal discs and was only moderately expressed in the CNS (see Fig. 4B; supplementary material Fig. S2). Likewise, expression of the mutated *let-7-Cp$^{3.3kb\Delta EcRE1\text{-}3}$::LacZ* was not detected in the ring gland and salivary glands, and was greatly diminished in prepupal leg and wing imaginal discs and to a lesser degree in the prepupal CNS, suggesting the presence of other temporally regulated tissue-specific enhancers in the 3.3 kb fragment. However, this fragment may also contain additional tissue-specific cryptic EcRE-like sequences that were not detected by our luciferase reporter assays. Though the expression of the *let-7-Cp$^{3.3kb\Delta EcRE1\text{-}3}$::LacZ* reporter appeared to be much stronger in –18 hour third instar larvae, a significant reduction in the staining pattern of some cells was observed in the CNS at puparium formation (0 hours) (Fig. 4B). A more drastic difference in expression of the *let-7-Cp$^{3.3kb}$::LacZ* and *let-7-Cp$^{3.3kb\Delta EcRE1\text{-}3}$::LacZ* was observed at later time points (Fig. 4C). These data indicate that the intron 1 EcREs play an important role in the onset and maintenance of *let-7-C* expression in a tissue-specific manner: EcRE-1 to EcRE-3 are essential *cis*-acting elements in tissues such as imaginal discs and salivary glands, but function along with additional enhancer elements in the CNS.

EcRE-1, EcRE-2 and EcRE-3 are bound by EcR/Usp

To obtain evidence that the EcR/Usp nuclear hormone heterodimer directly activated pri-let-7-C, we tested whether *let-7-C* EcREs could be bound by EcR/Usp using electrophoretic mobility shift assays (EMSAs). The migration of labeled DNA fragments encoding EcRE-1 to EcRE-3 were retarded when co-incubated with in vitro translated Usp protein and any of three EcR isoforms (EcR-A, EcR-B1 and EcR-B2), indicating that an EcR/Usp complex formed on each *let-7-C* EcRE (Fig. 3C, lanes 4,6,8,13,15,17,22,24,26). These complexes were supershifted by an antibody that recognized all EcR isoforms (anti-EcR-c) (Fig. 3C, lanes 5,7,9,14,16,18,23,25,27; supplementary material Fig. S4, lanes 3,8,13). Furthermore, complexes were completely abolished after incubation with an unlabeled wild-type EcRE but not a mutant EcRE competitor (Fig. 3A,D). To test whether endogenous EcR/Usp complexes could bind *let-7-C* EcREs, we performed analogous EMSA experiments using nuclear extracts from 20E-treated Kc-167 cells instead of in vitro translated proteins (supplementary material Fig. S4). We detected a similar complex that bound to all three EcREs (supplementary material

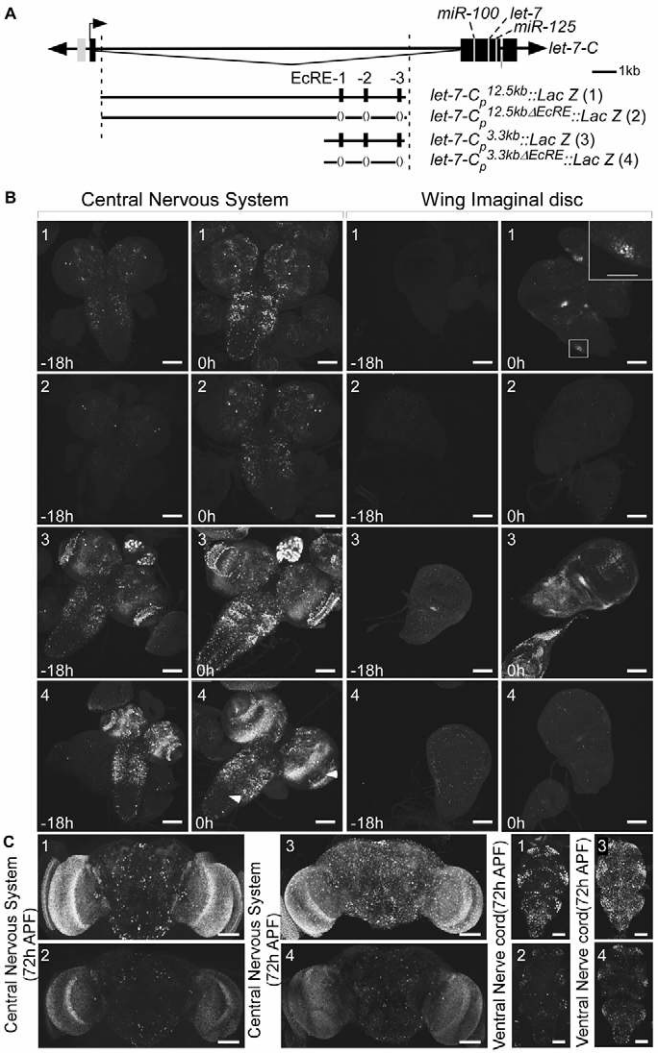

Fig. 4. *let-7-C* EcREs are required for tissue-specific induction and maintenance of pri-let-7-C reporters. (**A**) Schematic representation of *let-7-C* transcriptional reporters numbered 1 to 4: *let-7-Cp*$^{12.5kb}$*::LacZ* (1), *let-7-Cp*$^{12.5kb\Delta EcRE}$*::LacZ* (2), *let-7-Cp*$^{3.3kb}$*::LacZ* (3) and *let-7-Cp*$^{3.3kb\Delta EcRE}$*::LacZ* (4). (**B**) β-Galactosidase staining of central nervous system (CNS) and wing imaginal disc from wandering larvae (–18 hours APF) or white prepupae (0 hours) that harbored *let-7-C* intron transgenes 1-4. Transgene number is indicated in the top left of each panel, and animal stage is indicated in bottom left of each panel. The inset in the top right panel indicates a magnified view of a cluster of cells expressing the *lacZ* transgene in the imaginal disc. Arrowheads indicate absence of expression in subsets of CNS cells from transgene 4 containing white prepupae. Scale bars: 100 μm; 50 μm in inset. (**C**) β-Galactosidase staining of CNS and ventral nerve cord from 72 hours APF pupae that harbored *let-7-C* intron transgenes 1-4. Transgene number is indicated in the top left of each panel. Scale bars: 100 μm.

Fig. S4, lanes 2,7,12) and that was supershifted by the EcR-c antibody (supplementary material Fig. S4, lanes 3,8,13). The EcR-B1 antibody also supershifted the complex, whereas the EcR-A antibody had no effect (supplementary material Fig. S4, lanes 4,5,9,10,14,15), indicating that EcR B1-containing heterodimers bind more effectively to *let-7-C* EcREs in cells where all EcR isoforms are present. Together, these results demonstrate that the three *let-7-C* EcRE motifs are binding sites for EcR-Usp, and suggest that pri-let-7-C is direct target of this nuclear hormone receptor heterodimer.

EcR signaling is required for *let-7-C* expression

To obtain evidence that EcR is required for production of pri-let-7-C, we analyzed pri-let-7-C levels in EcR-deficient L57-3-11 cells. This Kc-167-derived cell line displays about 10% of the 20E-binding capacity of Kc-167 cells (Cherbas and Cherbas, 1997; Hu et al., 2003). The level of induction of pri-let-7-C was significantly reduced in L57-3-11 cells when compared with Kc-167 cells (Fig. 5A, top panel). Consequently, a negligible amount of processed let-7 miRNA was detected in L57-3-11, even after 48 hours of 20E treatment when compared with Kc-167 cells (Fig. 5A, bottom panel). Thus, reduction of EcR is associated with diminished pri-let-7-C expression.

To determine whether EcR regulates *let-7-C* expression via the EcRE regulatory motifs, we tested whether dominant-negative EcR isoforms could affect the 20E-responsiveness of the EcRE-1-containing 7-2 reporter construct described above (Fig. 2B). Consistent with a direct role for EcR in mediating the 20E induction of pri-let-7-C, we found that the increase in luciferase activity was reduced from ~37-fold to ~ 6-fold upon co-transfection of a dominant-negative EcR mutant, which competes with the endogenous EcR to block the normal hormonal response (Cherbas et al., 2003). 20E-mediated induction was rescued ~11-, 13- and 15-fold by co-transfection of EcR isoforms A, B1 and B2, respectively (Fig. 5B). In addition, co-transfection of EcR isoforms partially restored the hormone-inducible expression of the same luciferase reporter in the L57-3-11 EcR-deficient cell line: the 7-2 reporter displayed a ~4-fold induction of luciferase activity upon 20E treatment in L57-3-11 cells, and transfection of the different EcR isoforms A, B1 and B2 increased the luciferase activity by 7.8-, 7.4- and 12.3-fold, respectively (Fig. 5C). These data indicated that an EcRE within the *let-7-C* locus is required for EcR-mediated transcriptional activation of a luciferase reporter in cultured cells.

To examine whether EcR is required for *let-7-C* expression in vivo, the effect of dominant-negative EcR expression, as well as of EcR knockdown, on pri-let-7-C levels was measured in salivary glands. Dominant-negative (EcR-DN) and RNAi (EcR-RNAi) UAS transgenes targeting EcR were expressed in larval salivary glands using the salivary gland Gal4 driver (sgGal4) (Costantino et al., 2008), and semi-quantitative RT-PCR was performed to determine levels of pri-let-7-C in –18 hours, –8 hours, –4 hours and 0 hour APF RNA samples. Expression of EcR-DN or EcR-RNAi greatly diminished pri-let-7-C levels in third larval instar salivary glands (Fig. 5D). Moreover, expression of an EcR isoform in the EcR-DN genetic background completely restored the pri-let-7-C and E74A levels in salivary glands from third instar larvae (Fig. 5D). These data indicated that EcR is required for endogenous pri-let-7-C expression in salivary glands.

To confirm the cell-autonomous role of EcR in regulating pri-let-7-C expression, we tested whether expression of a *let-7-Cp*$^{3.3kb}$*::LacZ* transcriptional reporter was reduced in clones expressing either the EcR-DN or EcR-RNAi constructs (supplementary material Fig. S5). Tissues were dissected from late third larval instar and pupae (–8 to 12 hours APF). As expected, knock down of endogenous EcR function in salivary glands, imaginal discs and CNS clones abolished the expression of the *let-7-Cp*$^{3.3kb}$*::LacZ* reporter (Fig. 5E,F; data not shown). These results clearly demonstrated that 20E-coupled EcR signaling is required for the induction of pri-let-7-C in vivo.

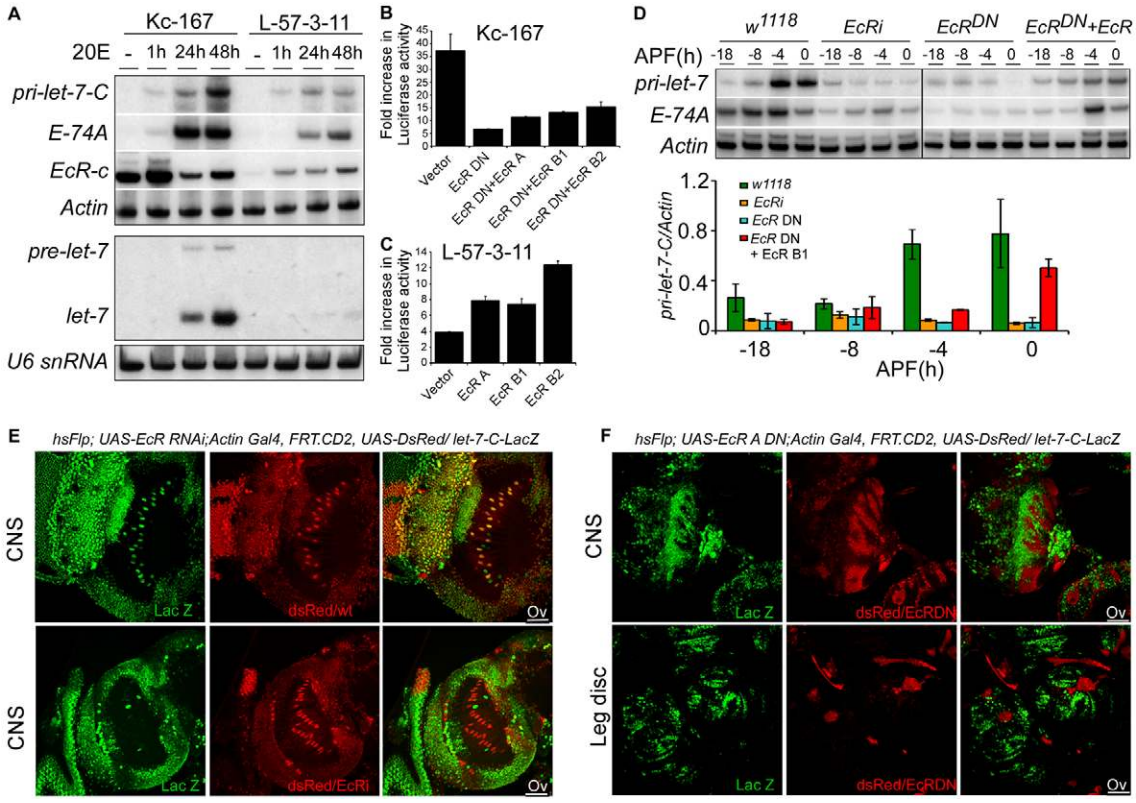

Fig. 5. EcR is required for pri-let-7-C expression. (**A**) Hormone-inducible pri-let-7-C production is compromised in the L57-3-11 EcR-deficient cell line. Kc-167 and L57-3-11 cells were treated with 5×10^{-6} M 20E for 1, 24 and 48 hours. Total RNA was extracted from both untreated (–) and treated cells (1 hour, 24 hours and 48 hours), and analyzed by semi-quantitative RT-PCR (for pri-let-7-C, EcR-c, E74A and actin; top panel) and northern blot hybridization (for let-7 and U6snRNA; bottom panel). (**B**) Fold increase in luciferase activity of 20E-treated Kc-167 cells co-transfected with a *let-7-C* EcRE 1 luciferase reporter (Fragment 7-2), a construct expressing dominant-negative EcR (EcR-DN), and EcR A, EcR B1, EcR B2 or empty vector expression plasmids. The fold induction of each construct (vertical axis) was measured as luciferase activity of 20E-treated versus untreated samples. Histograms indicate the results of three independent experiments. (**C**) Fold increase in luciferase activity of 20E-treated L57-3-11 cells co-transfected with a *let-7-C* EcRE 1 luciferase reporter (Fragment 7-2) and EcR A, EcR B1, EcR B2 or empty vector expression plasmids. The fold induction of each construct was measured as in B. The histogram plot from three independent experiments is represented. (**D**) Semi-quantitative RT-PCR analysis of pri-let-7-C, E74A and actin (loading control) RNA levels in salivary gland RNA extracted from *sgGal4/+* larvae (w^{1118}), *sgGal4/UAS-EcR-DN* larvae (EcR-DN), *sgGal4/UAS-EcR-RNAi* larvae (EcRi) or *sgGal4/UAS-EcR-DN;UAS-EcR* larvae (EcRDN+EcR). The levels of pri-let-7-C relative to actin5c were quantitated from three independent experiments and are represented as a histogram plot below the gel image. (**E**) β-Galactosidase immunostaining of a *let-7-Cp*$^{3.3kb}$*::LacZ* reporter in a mosaic prepupal CNS. The top row depicts dsRed-positive wild-type clones, and the bottom row depicts dsRed-positive clones containing *actinGal4*-driven UAS-EcR-RNAi. Nuclear expression of β-galactosidase is drastically reduced in EcR-RNAi-expressing clones (left bottom panel). An overlay of the left and middle panels is depicted in the right panel. (**F**) β-Galactosidase immunostaining of a *let-7-Cp*$^{3.3kb}$*::LacZ* reporter in a prepupal mosaic CNS (top panel) and imaginal disc (bottom panel) from prepupae. DsRed-positive cells contain *actinGal4*-driven UAS-EcR-DN. Nuclear expression of *lacZ* is drastically reduced in clones overexpressing EcR-DN (left panels). The right panel is an overlay of the left and middle panels. Scale bars: 50 μm.

let-7-C EcREs are required for *let-7-C* function in vivo

To examine the functional relevance of the *let-7-C* EcREs, we prepared transgenes in which let-7-C cDNA was placed under the control of either a wild-type or EcRE-deleted version of the 3.3 kb intronic enhancer. The resulting *let-7-Cp*$^{3.3kb}$*::cDNA* and *let-7-Cp*$^{3.3kbΔEcRE1\text{-}3}$*::cDNA* transgenes were inserted into identical chromosomal locations using the ϕC31 system, crossed into a *let-7-C*-null background, and resulting adult flies were subjected to northern blot analysis (Fig. 6A). The *let-7-Cp*$^{3.3kb}$*::let-7-C cDNA* flies displayed near wild-type levels of let-7 and miR-125 RNAs and 48% (±16%) of miR-100 levels relative to wild type. By contrast, deletion of the EcREs resulted in reduced *let-7-C* miRNA expression: let-7, mir-125 and miR-100 levels in *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kbΔEcRE1\text{-}3}$*::cDNA* transgenic flies were 25% (±3.5%), 34% (±11%) and 22% (±11%) of their levels in wild-type flies, respectively. In order to address the role of *let-7-C* EcREs in the precise induction of expression of *let-7-C* miRNAs, northern blot analysis was also performed with RNA extracted from third instar larvae (–8 hours APF), prepupae (0 hours APF) and pupal (24 hours APF) stages of the *let-7-Cp*$^{3.3kb}$*::cDNA* and *let-7-Cp*$^{3.3kbΔEcRE1\text{-}3}$*::cDNA* transgenic lines (Fig. 6B). A significant delay (~24 hours APF) in onset of expression of all three *let-7-C* miRNAs was observed in *let-7-Cp*$^{3.3kbΔEcRE1\text{-}3}$*::cDNA* relative to wild-type and *let-7-Cp*$^{3.3kb}$*::cDNA* transgenics (Fig. 6B). Although the timing of expression of *let-7-C* miRNAs was unaltered in *let-7-Cp*$^{3.3kb}$*::cDNA* transgenics, their levels were substantially lower than wild type. Thus, other enhancers that are not present in the 3.3 kb fragment function to maintain normal levels of *let-7-C* miRNAs. Consistent with the expression analysis in transgenic flies, *let-7-C*

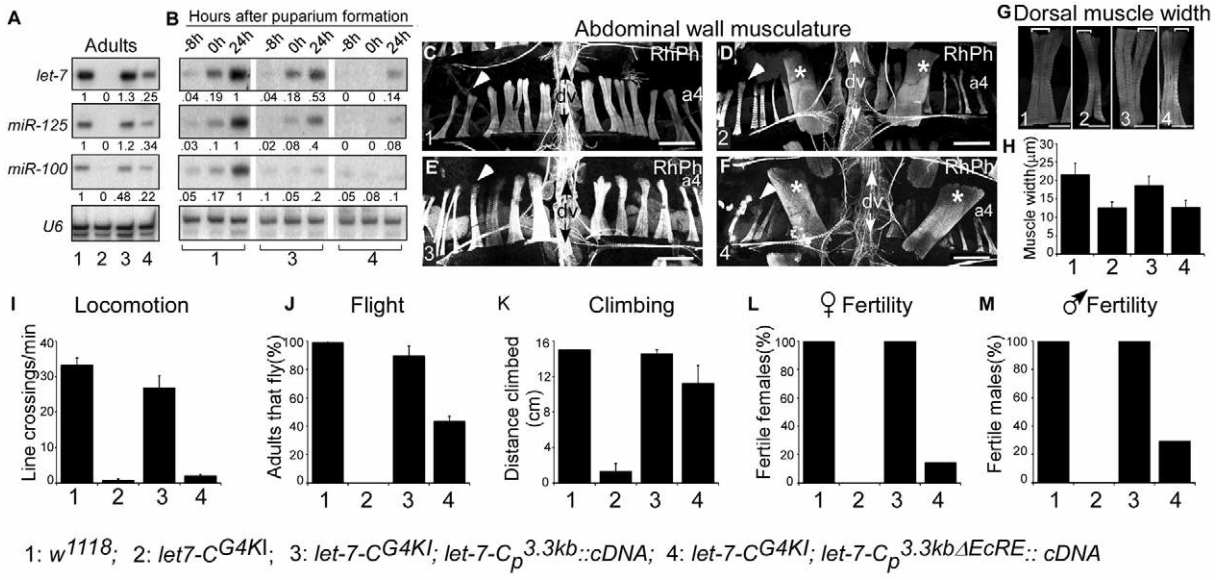

Fig. 6. let-7-C EcREs are required for *let-7-C* function in vivo. (**A**) Northern blot analysis of let-7-C miRNAs in three-day-old adult flies of the following genotypes: (1) w^{1118}, (2) *let-7-C*^{GKI}, (3) *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb}$*::cDNA* and (4) *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb\Delta EcRE}$*::cDNA*. Probes to detect let-7, miR-125 and miR-100 and U6snRNA were used for hybridization. The numbers underneath each band indicate the signal of the corresponding miRNA normalized to that of U6 snRNA presented relative to the expression in wild-type flies. (**B**) Developmental profile of mature let-7, miR-125 and miR-100 miRNAs, as detected by northern blot analysis of total RNA extracted from –8 hours, 0 hours and 24 hours APF using antisense let-7, miR-125 and miR-100 probes. U6snRNA was analyzed as a control for loading. Strains 1, 3 and 4 depicted in A were used for this analysis. The numbers underneath each band indicate the signal of the corresponding normalized miRNA relative to maximum expression of the corresponding miRNA in each panel. (**C-F**) The adult musculature of *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb\Delta EcRE}$*::cDNA* mutants display persistent pupal characteristics. Dorsal sections of abdominal segment 4 (a4) from 3-day-old w^{1118} (C), *let-7-C*^{GKI} mutant (D), *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb}$*::cDNA* (E) and *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb\Delta EcRE}$*::cDNA* (F) males stained with rhodamine phalloidin. The dorsal vessel (dv) runs along the dorsal midline in an anteroposterior orientation and bisects the a4 hemisegment. The *let-7-C*^{GKI} and *let-7-C*^{GKI}; *let-7-C*$_p$$^{3.3kb\Delta EcRE}$*::cDNA* flies display persistent DIOMs in each hemisegment (D and F, white asterisks). The adult DMs are indicated by white arrowheads (C-F). (**G**,**H**) The DMs in both *let-7-C*^{GKI} and *let-7-C*^{GKI}; *let-7-C*$_p$$^{3.3kb\Delta EcRE}$*::cDNA* flies are narrower than in both wild-type and *let-7-C*^{GKI}; *let-7-C*$_p$$^{3.3kb}$*::cDNA* flies. DMs from 3-day-old males labeled with rhodamine phalloidin (G, width indicated with brackets). (H) The histogram plot displays DM width measurement (n=40). (**I-M**) For all assays, the following genotypes were analyzed: w^{1118} in column 1, *let-7-C*^{GKI} in column 2, *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb}$*::cDNA* in column 3 and *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb\Delta EcRE}$*::cDNA* in column 4. Scale bars: 100 μm in C-F; 50 μm in G.

miRNAs were not induced in Kc-167 cells transfected with *let-7-Cp*$^{3.3kb\Delta EcRE1\text{-}3}$*::cDNA* construct (supplementary material Fig. S6). These data indicate that, although the EcREs are not totally responsible for *let-7-C* miRNA expression, they play a significant role in triggering and maintaining let-7, mir-125 and miR-100 expression.

To characterize the functional significance of this EcRE-mediated regulation of *let-7-C* miRNA expression in vivo, we tested the ability of *let-7-Cp*$^{3.3kb}$*::let-7-C cDNA* and *let-7-Cp*$^{3.3kb\Delta EcRE1\text{-}3}$*::cDNA* transgenes to rescue known *let-7-C* mutant phenotypes. Previous analysis has shown that the *let-7-C* locus is required for the remodeling of the neuromusculature during metamorphosis (Sokol et al., 2008). In *let-7-C* mutant adults, the pupal dorsal internal oblique muscles fail to decay, whereas the adult dorsal muscles do not achieve their normal size. We found that the *let-7-Cp*$^{3.3kb}$*::let-7-C cDNA* transgene rescued these phenotypes (Fig. 6E,G,H), whereas the EcRE mutated version did not (Fig. 6F-H). Furthermore, although *let-7-C*^{GKI} mutants that carried a *let-7-Cp*$^{3.3kb}$*::let-7-C cDNA* transgene were fully rescued for adult functions, the *let-7-C*^{GKI}; *let-7-Cp*$^{3.3kb\Delta EcRE1\text{-}3}$*::cDNA* transgenic flies displayed *let-7-C* mutant behavioral defects, including reduced motility, flight and fertility (Fig. 6I-M). Together, these data demonstrated that a minimal 3.3 kb fragment contained sufficient regulatory information to control endogenous *let-7-C* expression. Furthermore, the EcREs within this fragment are required to drive the *let-7-C* expression necessary for the formation of adult morphology and behavior.

DISCUSSION

Here, we present a series of data indicating that the *let-7-C* locus is a direct transcriptional target of EcR in vivo. We detect pri-let-7-C during the mid-third larval transition, a developmental stage when pulses of 20E activate the transcription of inducible genes through the EcR/Usp nuclear hormone heterodimer. We find that pri-let-7-C is rapidly induced in cultured *Drosophila* cells by 20E, and that the 20E responsiveness of the *let-7-C* locus requires EcR and is mediated by three 13-nucleotide EcR/Usp-binding sites. The deletion of these three EcREs eliminates *let-7-C* locus expression in larval and pupal tissues, including salivary glands and imaginal discs. EcRE deletion also causes a delay in *let-7-C* miRNA expression, as well as a reduction of *let-7-C* miRNA levels in adult flies, and is associated with known *let-7-C* mutant phenotypes. Taken together, these data strongly suggests that EcR binds to the endogenous *let-7-C* locus and activates its transcription in response to 20E, and that this transcriptional regulation is required for *let-7-C* miRNA function.

This work describes a clear convergence in the molecular mechanisms that control developmental timing in *Drosophila* and *C. elegans*. Members of the let-7 and miR-125 families of miRNAs

were originally identified in *C. elegans* as part of a pathway of heterochronic genes that promote stage-specific cell fate decisions (reviewed in Ambros, 2011). We have directly linked the fly orthologs of these heterochronic genes to the 20E/EcR pathway, which triggers stage-specific transcriptional cascades that direct major developmental transitions, including molting and metamorphosis (Thummel, 2001). We find that one essential function of the 20E/EcR pathway is to activate the expression of *let-7-C* miRNAs at the end of larval development to promote the formation of adult morphologies required for adult function.

Previous studies showing the slow onset of let-7 and miR-125 expression in tissue culture cells treated with Ecdysone had suggested that hormonal regulation of *let-7-C* expression may be indirect and not involve the 20E/EcR pathway (Sempere et al., 2002; Bashirullah et al., 2003; Sempere et al., 2003; Garbuzov and Tatar, 2010). We resolve this issue here, showing that pri-let-7-C is detected within 30 minutes of 20E treatment in an EcR-dependent fashion and thus is a likely direct target of 20E/EcR. The delayed expression of processed miRNAs previously observed might be due to the effects of Ecdysone on factors involved in the processing of pri-let-7-C, a possibility that has not been investigated in this study. An unresolved question, however, is why EcR knockdown initiated approximately 18 hours before puparium formation had no effect on the onset of let-7 and miR-125 expression, as described by Bashirullah (Bashirullah, 2003). Our interpretation is that let-7 and miR-125 were processed from pri-let-7-C transcripts that were already present when EcR levels were depleted in that experiment. Experiments presented here, including analysis of EcR-deficient cell lines and EcR-dominant negative transgenes, indicate that EcR is required for *let-7-C* expression in vivo.

Some nuclear hormone receptors both activate and repress the expression of direct targets. The *C. elegans* DAF-12 hormone receptor, for example, activates the *miR-241* promoter in the presence of DA steroid hormone, whereas it represses the *miR-241* promoter in the absence of DA (Bethke et al., 2009). Evidence supporting an analogous repressor function has been reported for EcR (Xu et al., 1999; Schubiger and Truman, 2000). Our results suggest that EcR solely functions to activate *let-7-C* expression in salivary gland and imaginal discs, as removal of EcREs 1-3 results in complete elimination, rather than derepression, of reporter expression in those tissues. The situation is a little less clear in the late larval CNS, as removal of the EcREs reduces but does not eliminate *lacZ* expression there. This EcRE-deleted reporter is not precociously expressed, though, suggesting that the EcRE sites are not required for reporter repression. We therefore suspect that *let-7-C* is co-activated in the CNS by EcR and at least one additional 20E-dependent factor. Indeed, the *Sgs3* and *Sgs4* salivary glue genes are controlled by complex ecdysone response units, which contain binding sites for other tissue-specific transcription factors such as the homeotic *forkhead* gene and potential binding sites for *Broad-Complex* gene (Lehmann and Korge, 1995; Crossgrove et al., 1996; Lehmann and Korge, 1996; Mach et al., 1996; Costantino et al., 2008).

A growing number of papers have suggested the functional orthology between the 20E/EcR and DA/Daf-12 pathways, as they play similar roles in regulating developmental timing, physiology, reproductive maturation and longevity in flies and worms, respectively (see Galikova et al., 2011; Tennessen and Thummel, 2011). These pathways not only play analogous functions but also share upstream components: the production of 20E as well as DA involves the TGFβ and IGF pathways. The work presented here suggests that they also share at least one common effector: *let-7-C* miRNAs. Steroid hormone regulation of *let-7-C* miRNAs may therefore represent an ancestral pathway that plays widespread roles both developmentally and post-developmentally

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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